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Phagocytosis of Apoptotic Neutrophils Regulates Granulopoiesis via IL-23 and IL-17

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Summary

Homeostatic regulation of neutrophil production is thought to match neutrophil elimination to maintain approximately constant numbers in the blood. Here, we show that IL-17, a cytokine that regulates granulopoiesis through G-CSF, is made by γδ T cells and unconventional αβ T cells. These neutrophil-regulatory T cells (Tn) are expanded in mice that lack leukocyte adhesion molecules, which have neutrophilia and defective neutrophil trafficking. Normal neutrophils migrate to tissues, where they become apoptotic and are phagocytosed by macrophages and dendritic cells. This curbs phagocyte secretion of IL-23, a cytokine controlling IL-17 production by Tn cells. Adoptive transfer of wild-type, but not adhesion molecule-deficient, neutrophils into mice deficient in β_2 integrins transiently decreases neutrophilia and reduces levels of serum IL-17. Antibody blockade of the p40 subunit of IL-23 reduces neutrophil numbers in wild-type mice. These findings identify a major homeostatic mechanism for the regulation of neutrophil production in vivo.

Introduction

Peripheral blood neutrophil numbers are tightly regulated in both mice and humans. Homeostatic regulatory mechanisms have been proposed (Demetri and Griffin, 1991) and debated (Horwitz et al., 2001) but never demonstrated. Enormous numbers of neutrophils (10^{11} polymorphonuclear neutrophils (PMN)/day in humans), which circulate for a few hours and undergo apoptosis, are produced every day. Older neutrophils in the blood start expressing CXCR4 and home back to the bone marrow (Martin et al., 2003); however, this process is unlikely to occur in neutrophils that have left the bloodstream and have migrated into tissues.

Phagocytosis of apoptotic cells triggers powerful anti-inflammatory signals (Savill and Fadok, 2000; Stuart et al., 2002). To investigate whether the phagocytosis of apoptotic neutrophils produces signals that regulate neutrophil production, we take advantage of mice deficient in leukocyte or endothelial adhesion molecules such as integrins or selectins. Most of these mice exhibit altered neutrophil trafficking and mild to severe neutrophilia (Frenette et al., 1996; Mayadas et al., 1993; Forlow et al., 2002; Scharffetter-Kochanek et al., 1998; Robinson et al., 1999; Ellies et al., 1998; Bullard et al., 1996; Forlow and Ley, 2001), which may compensate for reduced efficiency of adhesion and transmigration (Miyamoto et al., 2003; Jung et al., 1998). It is possible that these mice keep making more neutrophils until enough of them can enter critical target tissues.

The first plausible explanation for neutrophilia in these mice was the passive accumulation hypothesis. Neutrophil accumulation could be due to prolonged half-life or an inability to leave the vasculature (Coxon et al., 1996; Johnson et al., 1995; Weinmann et al., 2003). However, bone marrow chimeras produced in lethally irradiated wild-type (WT) mice reconstituted with mixed bone marrow from $CD18^{-/-}$ and WT mice show normal neutrophil numbers (Forlow et al., 2001; Horwitz et al., 2001), which excludes the possibility that $CD18^{-/-}$ neutrophils passively accumulate in the blood.

Alternatively, neutrophil homeostasis may be achieved in response to subclinical infection that results in chronic inflammation in adhesion molecule-deficient mice, which in turn causes neutrophilia. However, $CD18^{-/-}$ mice given a course of antibiotics did not show a significant reduction in blood neutrophil counts (Weinmann et al., 2003). In addition, the internal organs of $CD18^{-/-}$ and other mice have been screened without evidence of systemic infection (Forlow et al., 2001; Weinmann et al., 2003; Forlow et al., 2002). The absence of pathological bacteria does not exclude the possibility that products of normal commensal flora provide an important stimulus for normal neutrophil homeostasis. Here, we propose that an inhibitory feedback loop normally regulates granulopoiesis, and that this negative feedback loop is disturbed in adhesion molecule-deficient mice.

IL-17 is a proinflammatory cytokine expressed and secreted by T lymphocytes (Fossiez et al., 1996; Spriggs, 1997; Infante-Duarte et al., 2000; Yao et al., 1995b; Happel et al., 2003) that can induce G-CSF-dependent neutrophilia when expressed in mice (Schwarzenberger et al., 1998, 2000). It acts upstream of G-CSF to regulate granulopoiesis in adhesion molecule-deficient mice (Forlow et al., 2001). A newly discovered cytokine, IL-23, has been shown to stimulate IL-17 production in T lymphocytes (Aggarwal et al., 2003; Happel et al., 2003). IL-23 is a heterodimer containing two subunits, p19, which is unique to IL-23, and p40, which is shared with IL-12 (Oppmann et al., 2000). Interestingly, p19 transgenic mice display neutrophilia (Wiekowski et al., 2001). IL-23 is made by both dendritic cells (DCs) and macrophages (Mϕs) (Oppmann et al., 2000). We reasoned that apoptotic cell phagocytosis might downregulate IL-23 secretion.

Our working hypothesis was that neutrophils normally traffic to peripheral tissues, where they are phagocytosed by Mϕs and DCs after transmigration and apoptosis, which may downregulate IL-23 production by

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DCs and Mϕs. Reduced IL-23 would then curb IL-17 and G-CSF production and eventually granulopoiesis. If this process were interrupted by an inability of neutrophils to transmigrate due to adhesion molecule deficiency, tissue Mϕs and/or DCs would continue to express IL-23. This could drive IL-17 expression and increase granulopoiesis until enough neutrophils reach the peripheral tissues and close the feedback loop. Here, we present evidence that engulfment of apoptotic neutrophils regulates cytokine production by phagocytes to achieve neutrophil homeostasis.

Results

Identification of IL-17-Producing Cells

To identify the source of IL-17 in vivo, we first analyzed various organs from WT and adhesion molecule-deficient mice for their relative expression of IL-17 mRNA by real-time quantitative RT-PCR normalized to 18s RNA. IL-17 mRNA expression was found to be highest in WT mesenteric lymph node (MLN), followed by lung, terminal ileum (TI), jejunum, and spleen (Figure 1A). When stimulated MLN cells from WT mice were stained for intracellular IL-17, two populations of IL-17-producing cells were found, one expressing αβ-T cell receptor (TCR), and the other expressing γδ-TCR (Figure 1B). Blood neutrophil numbers are severely elevated in CD18$^{-/-}$ mice (Scharffetter-Kochanek et al., 1998) and in mice lacking both endothelial selectins, E- and P-selectin (E/P$^{-/-}$) (Bullard et al., 1996; Frenette et al., 1996). In both CD18$^{-/-}$ and E/P$^{-/-}$ double knockout mice, IL-17 mRNA is elevated in the spleen, lung, MLN, TI, and jejunum compared to WT mice (Figure 1C). Conversely, neutrophil numbers in the peripheral blood are reduced to 728 ± 56 PMN/μl (n = 8) in IL-17 receptor-deficient (IL-17R$^{-/-}$) mice compared to 1,145 ± 165 PMN/μl (n = 8) in WT mice ($p < 0.05$).

To investigate whether elevated IL-17 production is a general phenomenon in adhesion molecule-deficient mice, we surveyed mice with mild neutrophilia lacking only one of the leukocyte integrins, $\alpha_L\beta_2$ (LFA-1$^{-/-}$) (Ding et al., 1999), or core2-glucosaminyltransferase (Core2$^{-/-}$) (Ellies et al., 1998), an enzyme required for the biosynthesis of many selectin ligands. Lastly, we chose mice lacking all three selectins (E/P/L$^{-/-}$), which have an intermediate phenotype (Robinson et al., 1999; Collins et al., 2001). Figure 2 shows representative dot plots of gated lymphocytes from the spleens of CD18$^{-/-}$, E/P$^{-/-}$, E/P/L$^{-/-}$, LFA-1$^{-/-}$, Core2$^{-/-}$, and WT mice stained for γδ-TCR and αβ-TCR, which are expressed on T lymphocytes. Three distinct populations are defined as γδ T lymphocytes (R1), αβ T lymphocytes that are mostly CD4 negative and express intermediate amounts of αβ-TCR (αβ^{int}-TCR, R2), and αβ T lymphocytes that express high levels of αβ-TCR (αβ^{high}-TCR, R3). In WT mice with mild neutrophilia, the distinction between the R2 and R3 populations is less clear (Figure 2).

The R1 (second column of Figure 2, and data not shown) population of γδ T cells that express IL-17 are CD3^{high}, CD4^{-}, and CD8^{-}. The R2 (third column Figure 2, and data not shown) population consists of CD3^{int} CD8^{-} αβ^{int}-TCR lymphocytes that produce IL-17. They have an oligoclonal TCR distribution with about 60% Vβ8-TCR (data not shown), which is consistent with an NK T cell phenotype (Emoto and Kaufmann, 2003; MacDonald, 2002; Watanabe et al., 1995; Moodycliffe et al., 1999). However, they do not produce significant amounts of IFN-γ or IL-4 upon stimulation (data not shown) and do not express NK1.1 (data not shown). In mice with mild neutrophilia, like Core2$^{-/-}$ and LFA-1$^{-/-}$, most CD3^{int} αβ^{int} T cells that produce IL-17 express CD4 (Figure 2), but this is not the case in mice with severe neutrophilia, like CD18$^{-/-}$ or E/P$^{-/-}$, in which most IL-17-producing αβ^{int} T cells are CD4^{-} (Figure 2). E/P/L$^{-/-}$ mice display intermediate neutrophilia and intermediate levels of CD4^{+} expression on IL-17-producing αβ^{int} T cells (Figure 2). The R3 population (far right column Figure 2) that produces IL-17 includes conventional αβ T cells that are mostly CD3^{high}CD4^{+}CD8^{-}.

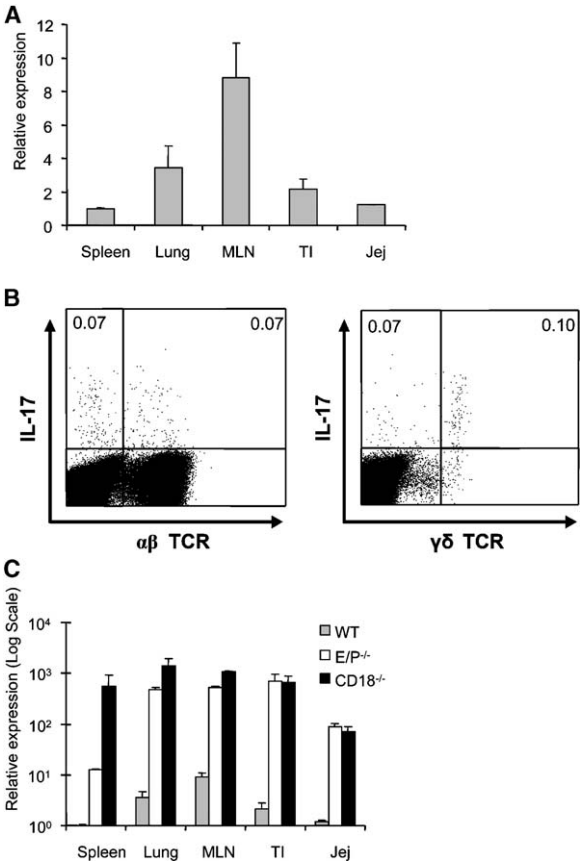

Figure 1. Real-Time Quantitative RT-PCR Reveals Localization of IL-17 Expression

(A) Spleen, lung, mesenteric lymph node, terminal ileum, and jejunum of WT mice were assayed for IL-17 mRNA expression by real-time quantitative RT-PCR. All values are normalized to 18s RNA. Results are shown as arbitrary units (WT spleen = 1).

(B) WT mesenteric lymph node cells were analyzed by flow cytometry after intracellular staining for IL-17 and surface staining for αβ- and γδ-TCR. Lymphocytes gated by forward versus side scatter were analyzed. The numbers in the upper quadrants indicate the percentage of all gated cells.

(C) IL-17 expression in tissues from E/P$^{-/-}$ (open bars) and CD18$^{-/-}$ (black bars) compared to WT mice (gray bars). Note the logarithmic scale.

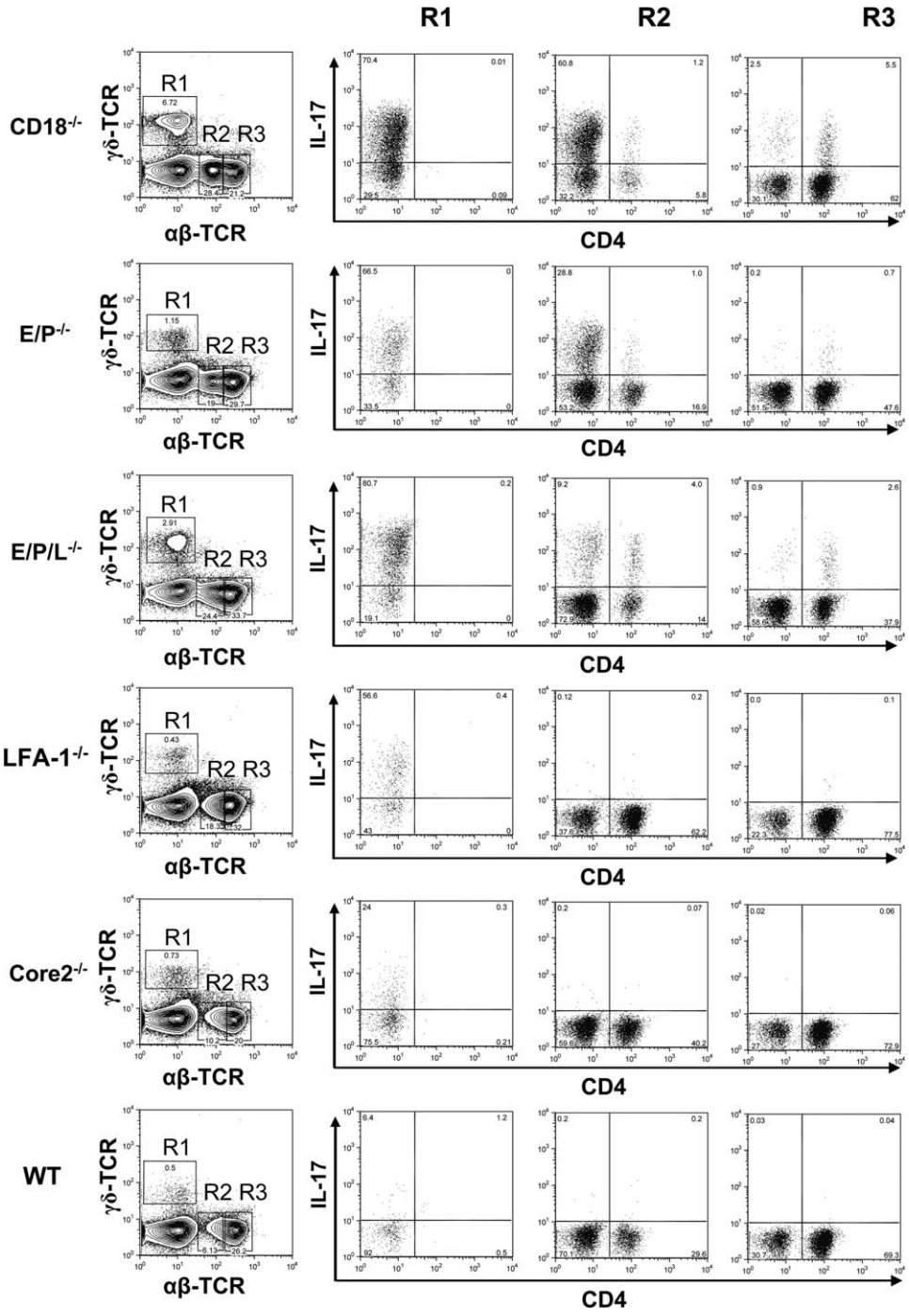

Figure 2. Expanded Subsets of γδ T Lymphocytes and NK T-like Lymphocytes Produce IL-17 in Adhesion Molecule-Deficient Mice

Splenocytes from CD18$^{-/-}$, E/P$^{-/-}$, E/P/L$^{-/-}$, LFA-1$^{-/-}$, Core2$^{-/-}$, and WT mice were analyzed by flow cytometry after intracellular staining for IL-17 and surface antigens. Three populations of T lymphocytes are gated R1, R2, and R3 (far left column) and then analyzed for CD4 and IL-17 expression (right three columns). Numbers in the corners of each panel represent the cell number as a percentage of all cells in the respective gate.

We find that all IL-17-producing cells are CD45RB^{low}, CD44^{high}, and CD62L^{low} (L-selectin) (data not shown), which is characteristic of effector memory cells (Watanabe et al., 1995; Sprent, 1997; Sprent and Surh, 2001; Oehen and Brduscha-Riem, 1998).

Table 1 shows a summary of all IL-17-producing cells in splenocytes of the six genotypes tested. For simplicity, αβ^{high} and αβ^{int} CD4^{-} cells are not shown separately. Even in normal C57BL/6 WT mice, some γδ T cells produce IL-17. The number of these cells increases steadily with increased neutrophilia. In mice with mild neutrophilia (Core2$^{-/-}$ and LFA-1$^{-/-}$) CD4^{+} αβ T cells constitute

Table 1. Subset Distribution of IL-17-Producing Tn Cells

	IL-17-Producing Cells as a Percentage of Total Lymphocytes				Percent of All IL-17-Producing Cells		
	All	γδ T Cells	αβ T Cells		γδ T Cells	αβ T Cells	
Genotype			$CD4^+$	$CD4^-$		$CD4^+$	$CD4^-$
$CD18^{-/-}$	22 ± 2	5 ± 2	1.8 ± 0.04	15 ± 3	24 ± 11	8 ± 1	68 ± 11
$E/P^{-/-}$	4 ± 2	0.7 ± .2	0.4 ± 0.1	3 ± 1	15 ± 3	10 ± 2	75 ± 4
$E/P/L^{-/-}$	4 ± 2	1 ± .9	1.2 ± 0.6	1 ± 0.7	22 ± 10	34 ± 7	43 ± 5
$LFA\text{-}1^{-/-}$	0.7 ± 0.6	0.2 ± 0.1	0.4 ± 0.4	0.1 ± 0.1	47 ± 22	39 ± 15	13 ± 6
$Core2^{-/-}$	0.5 ± 0.4	0.1 ± 0.1	0.3 ± 0.3	0.1 ± 0.1	31 ± 23	59 ± 22	10 ± 5
Wild-type	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.01 ± 0.00	63 ± 3	33 ± 2	3.9 ± 0.2

Percent ± SEM of spleen lymphocytes determined by forward and side-scatter properties from at least three separate experiments. The $CD4^-$ subset of Tn cells includes $\alpha\beta^{high}$ and $\alpha\beta^{int}$ T lymphocytes.

more than 70% of the αβ T cells that produce IL-17. In $CD18^{-/-}$ mice, the most important IL-17-producing T cells are $CD4^-$ $CD3^{int}$ αβ-TCR^{int} T cells.

Phagocytosis of Apoptotic Neutrophils Decreases IL-23 Secretion in Bone Marrow-Derived Dendritic Cells and Macrophages

One of the cytokines regulating IL-17 production is IL-23. IL-23 is predominantly made by DCs and Mϕs (Oppmann et al., 2000) and induces IL-17 secretion in cultured T cells in vitro (Aggarwal et al., 2003; Happel et al., 2003). We investigated whether IL-23 would stimulate IL-17 production in the subset of γδ and αβ T cells that produce IL-17. We took advantage of splenocytes from $CD18^{-/-}$ mice, which spontaneously have large numbers of IL-17-producing cells. Unfractionated $CD18^{-/-}$ splenocytes (2 × 10^6 cells/ml) were incubated with either rIL-23 or rIL-12 for 24 hr, and IL-17 concentration in the supernatant was measured by ELISA. rIL-23, but not rIL-12, stimulated IL-17 production in a dose-dependent manner (Figure 3A).

To investigate the physiology of IL-17 regulation, unfractionated $CD18^{-/-}$ splenocytes were cocultured with LPS-stimulated bone marrow-derived dendritic cell (BMDC)-conditioned media for 24 hr with or without an antibody against the common subunit to IL-12 and IL-23 (p40) or an antibody specific to IL-12 (p70) (Figure 3B). In agreement with a previously published report

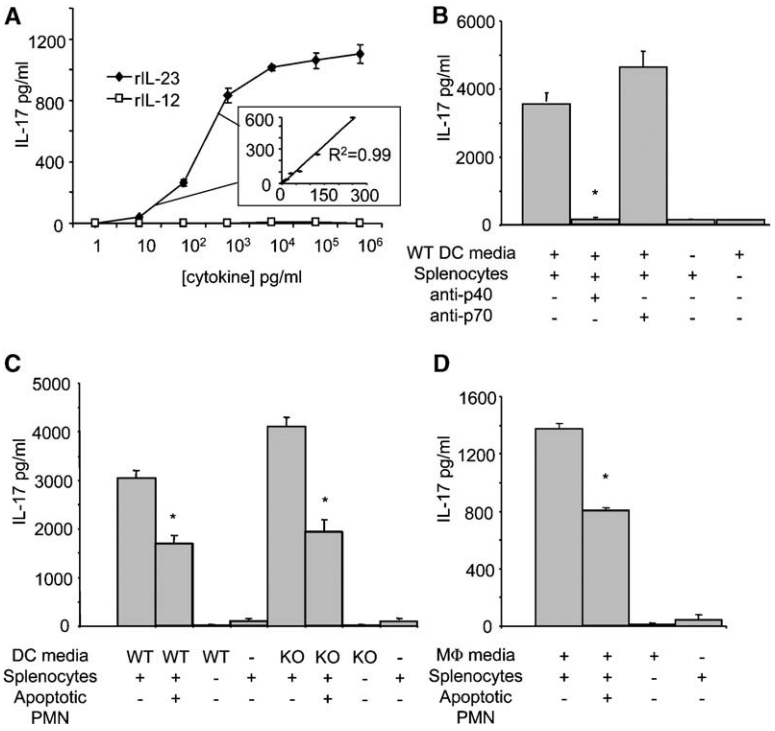

Figure 3. IL-23 Production in DCs and Mϕs Is Regulated by Phagocytosis of Apoptotic Neutrophils

$CD18^{-/-}$ splenocytes were used as a bioassay to detect IL-23 in cell culture-conditioned media.

(A) $CD18^{-/-}$ splenocytes were incubated with either rIL-23 or rIL-12 for 24 hr at 37°C, and supernatants were assayed for IL-17 by ELISA.

(B) BMDCs were stimulated for 24 hr with 100 ng/ml LPS. Cell-free conditioned media were added to $CD18^{-/-}$ splenocytes (2 × 10^6/ml final concentration). Conditioned media of BMDCs alone contain no detectable IL-17. WT BMDC-conditioned media cultured with $CD18^{-/-}$ splenocytes stimulate IL-17. This effect is inhibitable by preincubation of BMDC-conditioned media with mAb to the p40 subunit (anti-p40) common to both IL-23 and IL-12. Preincubation with an IL-12-specific mAb (anti-p70) has no effect (n = 4); *p < 0.05. Error bars show mean ± SEM.

(C) WT BM was aged in complete media for 8 hr, resulting in 20% $GR\text{-}1^+$ apoptotic neutrophils as defined by annexin V staining. Aged BM cells were added to WT or $CD18^{-/-}$ BMDCs in a 1:1 ratio and were allowed to interact overnight. They were then stimulated with 100 ng/ml LPS for 24 hr, and supernatant was assayed to determine IL-23 secretion as detected by its ability to stimulate IL-17 secretion in $CD18^{-/-}$ splenocytes. Results are representative of at least three separate experiments; *p < 0.05. Error bars show mean ± SEM.

(D) Aged apoptotic BM cells were added in a 3:1 to 2:1 ratio to peritoneal macrophages and allowed to interact for 2 hr. Nonphagocytosed cells were washed off. 100 ng/ml LPS was added, and the cells were incubated for 24 hr. Cell-free conditioned media were assayed for IL-23-induced IL-17 secretion. Results are representative of two separate experiments; *p < 0.05. Error bars show mean ± SEM.

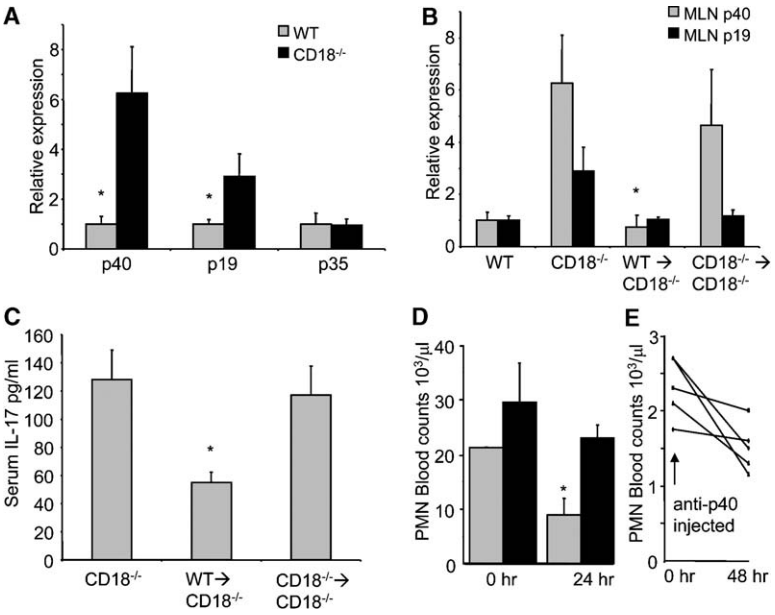

Figure 4. Adoptive Transfer of WT BM Neutrophils to CD18$^{-/-}$ Mice Reduces Elevated IL-23 Expression and Serum IL-17

(A) The mesenteric lymph nodes of WT and CD18$^{-/-}$ mice were assayed for p40, p19, and p35 mRNA expression by real-time quantitative RT-PCR. All values are normalized to 18s RNA. Results are shown as arbitrary units (WT expression = 1) (n = 3; *p < 0.05). Error bars show mean ± SEM.

(B) Aseptically isolated and purified BM-PMN cells from WT mice or CD18$^{-/-}$ (10^7/ml) were injected via tail vein (500 µl, 5 × 10^6 cells total) into CD18$^{-/-}$ mice. p40 and p19 expression in the mesenteric lymph node of recipient CD18$^{-/-}$ mice was assayed by real-time quantitative RT-PCR 24 hr after adoptive transfer (n = 3, *p < 0.05). Error bars show mean ± SEM.

(C) Serum IL-17 (pg/ml) was measured by ELISA 24 hr after adoptive transfer (n = 3, *p < 0.05). Error bars show mean ± SEM.

(D) Peripheral blood neutrophil counts of CD18$^{-/-}$ mice receiving WT unfractionated BM cells (gray bars) or control CD18$^{-/-}$ unfractionated BM cells (black bars) (n = 4, *p < 0.05). Error bars show mean ± SEM.

(E) WT mice injected with anti-p40 antibody showed reduced blood neutrophil counts 48 hr after mAb injection.

(Aggarwal et al., 2003; Happel et al., 2003), only antibody to the p40 subunit inhibited IL-17 production. These findings indicate that a p40-containing factor, most likely IL-23, is a major regulator of IL-17 production. In addition, p40 homodimers could also have biological effects and contribute to our observations.

IL-12 secretion is known to be downregulated when BMDCs phagocytose apoptotic cells (Stuart et al., 2002). To test the effect of phagocytosis of apoptotic neutrophils on IL-23 secretion, we measured IL-23 activity in conditioned media by its ability to induce IL-17 secretion of CD18$^{-/-}$ splenocytes. This bioassay showed a linear relation with the amount of IL-23 added (R^2 = 0.99). Very little IL-17 is produced from either unstimulated or LPS-stimulated CD18$^{-/-}$ splenocytes in control media, (30 ± 13 and 235 ± 32 pg/ml IL-17, respectively, versus over 3000 pg/ml in response to LPS-stimulated BMDC-conditioned media). Coculture of WT or CD18$^{-/-}$ BMDCs and Mϕs with apoptotic neutrophils decreased IL-23 secretion by about 50% (Figures 3C and 3D). The use of CD18$^{-/-}$ BMDCs demonstrates that phagocytes do not require β_2 integrins for efficient phagocytosis of apoptotic cells, consistent with previous reports with human macrophages (Ren et al., 2001). There is no published evidence that efficient phagocytosis requires the presence of β_2 integrins on apoptotic cells. To control for a secreted factor from apoptotic cells that would downregulate IL-23 secretion from BMDCs, we sorted fluorescently labeled WT or CD18$^{-/-}$ BMDCs into two populations that had phagocytosed fluorescently labeled apoptotic cells or not (data not shown). Only BMDCs that had ingested apoptotic cells showed marked reduction in IL-23 production by 62% and 56%, for WT and CD18$^{-/-}$ BMDCs, respectively.

Adoptive Transfer of Bone Marrow Neutrophils Transiently Normalizes Neutrophilia

Since IL-17 expression as determined by real-time RT-PCR is elevated in the MLN of CD18$^{-/-}$ mice compared to WT mice (Figure 1C), we predicted that IL-23 expression would be elevated as well. Real-time RT-PCR shows that both p40 and p19 (IL-23), but not p35 (IL-12), are significantly elevated in CD18$^{-/-}$ mice (n = 3; Figure 4A). Based on previous work with chimeric bone marrow reconstitution experiments of lethally irradiated mice (Forlow et al., 2001; Horwitz et al., 2001), we hypothesized that normal transmigration of WT neutrophils into peripheral tissues of adhesion molecule-deficient mice may restore neutrophil homeostasis. To test whether WT neutrophils would become phagocytosed, reduce IL-23, and consequently reduce IL-17, G-CSF, and neutrophil production, 5 × 10^6 WT or CD18$^{-/-}$ Percoll gradient-purified bone marrow neutrophils (BM-PMN > 95% neutrophils, GR-1^{high}) were injected via the tail vein into CD18$^{-/-}$ mice. WT, but not CD18$^{-/-}$ adoptively transferred BM-PMN, significantly decreased p40 expression in the MLN of CD18$^{-/-}$ recipient mice to WT levels of expression at 24 hr (n = 3; Figure 4B). p19 expression was marginally decreased. Serum IL-17 concentration was reduced by 52% ± 6% in CD18$^{-/-}$ mice that had been adoptively transferred with WT, but not CD18$^{-/-}$, BM-PMN. WT mice have undetectable serum IL-17 (<10 pg/ml). This finding suggests that WT neutrophils can transiently correct the neutrophilia seen in CD18$^{-/-}$ mice, presumably because they can reach the critical target organs, become phagocytosed, and suppress IL-23 and IL-17 levels. Consistent with reduced IL-17 levels, neutrophil counts in the blood of CD18$^{-/-}$ mice receiving WT cells were reduced at 24 hr (Figure 4D). This finding was confirmed by adoptive transfer of WT BM-PMN into mice that lack both α(1,3)-

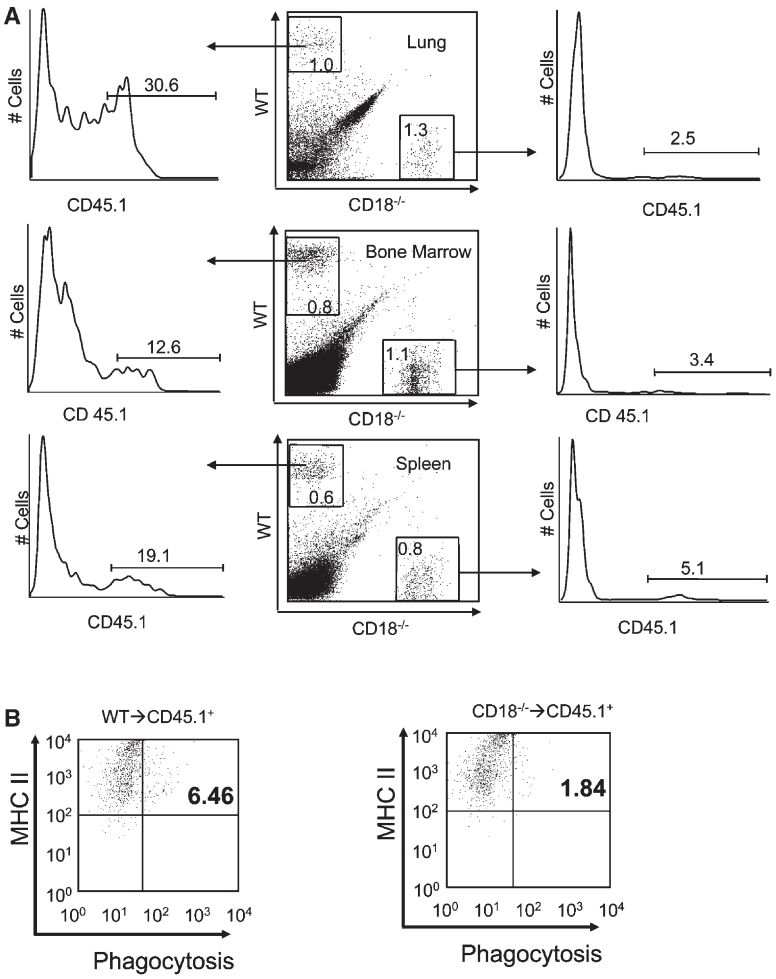

Figure 5. Adoptively Transferred WT, but Not CD18$^{-/-}$ BM Neutrophils, Reach Tissue DC and/or Mϕ to Be Phagocytosed

(A) Aseptically isolated BM cells from WT and CD18$^{-/-}$ mice were labeled with PKH26 and CFSE, respectively. A 1:1 ratio of cells (500 μl, 10^7 total cells) was injected via tail vein into a CD45.1$^+$ host. After 8 hr, single-cell suspensions of lung, bone marrow, and spleen were analyzed by flow cytometry. A CD45.1 host was used to evaluate phagocytosis of donor cells. The CD45.2$^-$, CD45.1$^+$ host cells will only be positive for PKH26 or CFSE after phagocytosis of fluorescently labeled donor cells. PKH26-labeled WT BM cells (left panel) or CFSE-labeled CD18$^{-/-}$ BM cells (right panel) were gated and analyzed for CD45.1 expression. Data are representative of two different experiments.

(B) Aseptically isolated BM-PMN cells from WT or CD18$^{-/-}$ mice (5 × 10^6/500 μl) were labeled with CFSE and injected via tail vein into CD45.1$^+$ recipient mice. After 4 hr, the MLN were harvested and treated with a collagenase-containing enzyme cocktail and evaluated by flow cytometry. Cells were first gated for CD45.1$^+$ host cells, then for DC by CD11c and MHC II expression. Phagocytosis can be determined by green fluorescence (CFSE-labeled BM-PMN).

fucosyltransferases (FucT)-IV and FucT-VII (Homeister et al., 2001), enzymes necessary for the generation of leukocyte ligands for E- and P-selectins. 24 hr after adoptive transfer, circulating neutrophil numbers were reduced by 40% to 18.8 ± 1.7 (10^3 PMN/μl). Infusing FucT IV/VII$^{-/-}$ BM-PMN had no such effect (30.8 ± 2.0 10^3 PMN/μl, n = 3, $p < 0.007$).

Immunoneutralization of IL-23

To test the validity of our proposed mechanism in WT mice, we neutralized IL-23 with an antibody to p40 i.p. Anti-IL-12 p40 mAb (C17.8) neutralizes p40-containing factors, including IL-12, IL-23, and p40 homodimers, but anti-IL-12p70 (C18.2) neutralizes only IL-12. Blood neutrophil counts were determined before and 48 hr after injection. Mice treated with anti-p40 antibody (C17.8) showed a significant decrease of blood neutrophil numbers from 2.31 ± 0.13 to 1.51 ± 0.05 (10^3 PMN/μl) (Figure 4E; n = 5, $p < 0.04$, paired t test), while those treated with anti-p70 antibody (C18.2) did not (average 2.5 ± 0.28 and 2.08 ± 0.12 10^3 PMN/μl, respectively).

In Vivo Trafficking of Neutrophils

In order to directly demonstrate phagocytosis of neutrophils in vivo, we injected via tail vein fluorescently labeled CD18$^{-/-}$ (CFSE) and WT (PKH26) unfractionated BM cells (both CD45.2$^+$) into the same CD45.1$^+$ mouse. PKH26 and CD45.1 double-positive events represent WT neutrophils ingested by recipient phagocytes, whereas CFSE-CD45.1 double-positive events represent CD18$^{-/-}$ neutrophils ingested by recipient phagocytes. WT cells were much more likely to be closely associated with CD45.1$^+$ phagocytes than CD18$^{-/-}$ neutrophils. This effect was significant in all organs tested. Association (phagocytosis) was reduced from 30.6% to 2.5% in the lung, 12.6% to 3.4% in the bone marrow, and 19.1% to 5.1% in the spleen when β_2 integrins were absent. Intact trafficking of CD18$^{-/-}$ neutrophils to the lung, bone marrow, and spleen indicates that CD18$^{-/-}$ neutrophils can enter these organs.

Since unfractionated bone marrow cells contain only 35%–60% mature neutrophils, we repeated similar experiments with isolated purified BM-PMN (>95% pure). When CFSE-labeled WT BM-PMN were injected into CD45.1$^+$ WT mice, 6.5% of all CD11c^{high} MHC II$^+$ DCs were closely associated with or had phagocytosed CFSE-labeled neutrophils at 4 hr after injection. By contrast, only 1.9% of MLN DCs were closely associated with or had phagocytosed CD18$^{-/-}$ BM-PMNs 4 hr after adoptive transfer (Figure 5B).

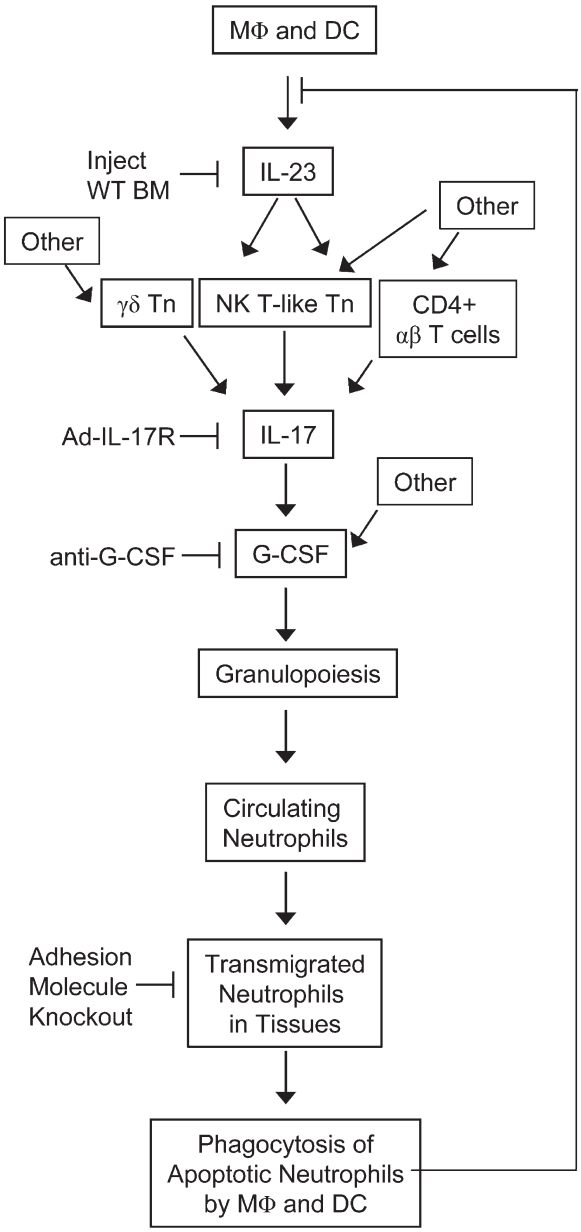

Figure 6. Model of IL-23/IL-17-Dependent Granulopoiesis

Normal neutrophils are able to adequately transmigrate into tissues. Apoptotic neutrophils are phagocytosed by Mϕ and DC, which reduces IL-23 production and resulting IL-17 production in $\gamma\delta$ Tn, NK T-like Tn, and CD4 T cells. Neutrophils produced in adhesion molecule-deficient mice are unable to efficiently transmigrate into critical peripheral tissue sites. Therefore, Mϕs and DCs that produce IL-23 do not phagocytose enough neutrophils that have become apoptotic. Increased IL-23 subsequently stimulates IL-17, G-CSF, and granulopoiesis until enough neutrophils are made to reach all critical target tissues. IL-17 increases G-CSF, which can be blocked by an adenovirus encoding for soluble IL-17 receptor (Forlow et al., 2001). G-CSF can be blocked by anti-G-CSF (Forlow et al., 2001), which directly regulates circulating neutrophils. "Other" indicates that other modifying factors likely exist at these levels. The line with the bar indicates inhibition.

Discussion

We used in vitro and complementary in vivo studies to reveal the mechanisms involved in neutrophil homeostasis. Our data suggest that phagocytosis of apoptotic neutrophils is more than just waste disposal. It also actively regulates neutrophil production by suppressing IL-23 secretion from phagocytes, which in turn reduces IL-17 and G-CSF. Elevated expression and secretion of IL-23 and IL-17 in CD18$^{-/-}$ mice is corrected by infusion of WT neutrophils. Two distinct populations of IL-17-producing cells, a $\gamma\delta$ T cell subset and an NK T cell-like subset, in addition to the previously identified conventional CD4$^+$ T cells are consistently found in five independent lines of adhesion molecule knockout mice with impaired neutrophil recruitment. Previous studies have shown that IL-17 production drives G-CSF (Forlow et al., 2001; Schwarzenberger et al., 1998; Schwarzenberger et al., 2000) and that G-CSF is directly responsible for the differentiation and proliferation of neutrophil precursors in the bone marrow (Lieschke et al., 1994; Demetri and Griffin, 1991; Richards et al., 2003). The present data close the missing links and complete the neutrophil homeostatic feedback loop (Figure 6).

IL-17-Producing Cells

IL-17 has been shown to produce neutrophilia when overexpressed in mice (Schwarzenberger et al., 1998) and is elevated in many inflammatory conditions (Miossec, 2003). The traditional view is that IL-17 is produced by activated CD4$^+$ T cells (Spriggs, 1997; Yao et al., 1995b; Infante-Duarte et al., 2000; Fossiez et al., 1996). We confirm that conventional CD3^{high}CD4$^+\alpha\beta$-TCR^{high} T cells with an activated phenotype produce IL-17 in splenocytes of mice with defects in neutrophil trafficking. However, a separate population of IL-17-producing CD4$^-$ $\alpha\beta$ T cells in the splenocytes of the six strains of mice examined shows intermediate $\alpha\beta$-TCR and CD3 expression, in addition to oligoclonal expression of Vβ8 (60%) similar to that found in NK T cells, although they do not express NK1.1, or produce IFN-γ or IL-4 upon stimulation. These cells are NK T-like unconventional $\alpha\beta$ cells. IL-17-producing NK T-like cells can be characterized as $\alpha\beta$-TCR^{int} Vβ8$^+$ CD3^{int} CD4$^-$ CD8$^-$ CD45RB^{lo} CD44^{high} CD62L^{low}. In WT mice, an even larger number of IL-17-producing cells are $\gamma\delta$ T cells that can be characterized as $\gamma\delta$-TCR$^+$ CD3^{hi} CD4$^-$ CD8$^-$ CD45RB^{lo} CD44^{high} CD62L^{low}. Together, these two newly identified cell populations comprise the majority of all IL-17-producing cells in the spleens of five strains of adhesion molecule knockout mice and normal C57BL/6 WT mice. We propose to call these cells "neutrophil regulatory T cells," or "Tn" cells. Perhaps it would be reasonable and convenient to distinguish between $\gamma\delta$ Tn and NK T-like Tn cells.

Anti-Inflammatory Effects of Phagocytosis of Apoptotic Neutrophils

During the resolution phase of inflammation, apoptosis is considered the "safe" and anti-inflammatory pathway of cell elimination (Savill and Fadok, 2000). Indeed, phagocytosis of apoptotic neutrophils has previously been shown to reduce secretion of IL-12 from dendritic

cells (Stuart et al., 2002). IL-23 was discovered only recently (Oppmann et al., 2000) and recognized to drive IL-17 production in mice. Here, we show that IL-23 and the resulting IL-17 production is elevated in CD18$^{-/-}$ mice, and both are reduced when WT apoptotic neutrophils are phagocytosed. The signaling pathways leading to reduced production of inflammatory cytokines have been explored by others (Ravichandran, 2003). The physiologic relevance of the observation of a regulatory role of phagocytosis of apoptotic neutrophils is underscored by our ability to suppress IL-23 (p40) expression, serum IL-17 levels, and neutrophilia by adoptively transferring WT neutrophils into CD18$^{-/-}$ mice.

Other Inputs

The homeostatic mechanism proposed in Figure 6 illustrates only the main regulatory pathway. We have shown that inhibitory interventions at critical checkpoints interfere with neutrophilia produced by the absence of adhesion molecules that are relevant to neutrophil trafficking. Although adoptive transfer of WT neutrophils to CD18$^{-/-}$ mice reduced p40 levels to normal, neutrophilia and serum IL-17 were only partially reduced. It is possible that IL-23 protein is still present 24 hr after adoptive transfer when p40 mRNA levels are normal. In addition, other modifying inputs are likely to exist at each stage of the regulatory feedback loop, which is really embedded in a large meshwork of positive and negative feedback circuits.

There are at least six isoforms of IL-17 and two receptors that have been shown to be physiologically relevant, IL-17R and IL-17RH1 (Moseley et al., 2003; Yao et al., 1995a). Both ligands of IL-17R, IL-17A (as measured here) and IL-17F, can induce granulopoiesis. Other members of the IL-17 family that have been shown to induce neutrophilia are IL-17B and IL-17C, which can bind IL-17RH1 (Hurst et al., 2002; Li et al., 2000). Mice deficient in IL-17A or its receptor are reported to have normal blood neutrophil levels (Nakae et al., 2002; Ye et al., 2001; Spriggs, 1997). However, we measured a significant 40% decrease of blood neutrophil numbers in IL-17R$^{-/-}$ mice as compared to WT mice.

Both G-CSF$^{-/-}$ or G-CSFR$^{-/-}$ mice exhibit severe neutropenia. These mice have mature neutrophils in the blood but only at 20% of the levels of WT mice (Lieschke et al., 1994; Liu et al., 1996). Additional deletion of IL-6 (G-CSFR × IL-6-deficient) results in a more severe phenotype (Liu et al., 1997), although IL-6-deficient mice have normal basal granulopoiesis. Thus, IL-6 is partially compensating for the G-CSF receptor deletion. Although acute immunoneutralization of IL-23 reduces blood neutrophil counts in WT mice, IL-23 (p19$^{-/-}$) mice have normal numbers of CD16^{+} cells, which include neutrophils, in the blood (Ghilardi et al., 2004). Similarly, p40$^{-/-}$ mice that lack both IL-23 and IL-12 have normal blood neutrophil counts (data not shown and Magram et al., 1996). These findings suggest that IL-23, which is relatively upstream in the regulatory mechanism, can be compensated for by other regulatory inputs. By contrast, mice lacking IL-17R have overt neutropenia, and mice lacking G-CSF or G-CSFR have very severe neutropenia, suggesting that the regulating mechanisms downstream of IL-23 are critical for normal neutrophil homeostasis.

Neutrophil Turnstile

In the context of the newly discovered feedback loop, the image of a turnstile counting the neutrophils as they come through is useful. Such a turnstile was first proposed to exist by Griffin's work (Demetri and Griffin, 1991), but was neither located nor defined at the cellular or molecular level. It is clear that the turnstile is not located at the exit point from the bone marrow, because neutrophil release from the bone marrow is not defective in adhesion molecule-deficient mice (Horwitz et al., 2001). It is also hard to conceive of a turnstile in the flowing blood. Our data suggest that the most important turnstile is located in the gastrointestinal tract, because, in WT mice, most IL-17 is expressed in the MLN. The small bowel is a place where the immune and inflammatory systems are confronted with antigens from endogenous bacterial flora, which may provide stimuli for the dendritic cells and macrophages to elaborate IL-23. Consistent with this concept, germ-free rats have neutrophil levels that are one-tenth of those of normal conventional rats (Ohkubo et al., 1999), suggesting that bacteria or bacterial products are necessary for normal circulating levels of neutrophils. Two recent publications support our hypothesis that normal flora in the gut is indeed necessary for proper neutrophil homeostasis. First, Becker et al. (2003) demonstrated that p40 (IL-23) expression in dendritic cells of the terminal ileum was dependent on bacterial stimulation in WT mice. Second, Macpherson and Uhr (2004) showed that dendritic cells from the Peyer's patch constitutively phagocytose commensal bacteria and traffic to the MLN. We speculate that neutrophil phagocytosis may occur in the Peyer's patch, lamina propria, and submucosal layers of the intestine, from where dendritic cells migrate to the MLN. It is possible that regulatory processes occur at other interfaces between epithelial tissues and the environment, such as the skin and lung. In fact, we found IL-17-expressing T cells in the mediastinal and peribronchial lymph node, suggesting that the homeostatic mechanism is not restricted to the intestinal system (data not shown).

Conclusions

We have tested our hypothesis most rigorously in CD18$^{-/-}$ mice, but we have also investigated WT and five other strains of adhesion molecule knockout mice and found results consistent with our model. Interestingly, serum IL-17 correlates nicely with the level of neutrophilia in each strain (Forlow et al., 2001), underscoring the wide applicability of this mechanism. However, we cannot exclude that in some knockout mice other regulatory pathways may exist. As predicted by our observation of negative regulation of IL-23 and subsequent IL-17 production by uptake of apoptotic neutrophils, we find very few IL-17-producing T cells in normal WT mice under specific pathogen-free conditions. We believe this reflects the normal suppressive effect of continuous uptake of apoptotic neutrophils by tissue dendritic cells and macrophages. A few IL-17-express-

ing cells are found in the MLN of WT mice, and they show characteristics of γδ T and NK T-like cells.

Our data establish a physiologic feedback circuit that regulates neutrophil homeostasis in mice and identifies the IL-23 – IL-17 – G-CSF axis as the major regulatory pathway. Phagocytosis of apoptotic cells by dendritic cells and macrophages represents the critical inhibitory step, which is central for a negative feedback loop. The discovery of neutrophil-regulating γδ and NK T-like cells suggests new ways to manipulate the inflammatory system and neutrophil production with possible relevance to the treatment of inflammatory diseases and the mitigation of chemotherapy-induced neutropenia.

Supplemental Data

Supplemental Data including detailed Experimental Procedures are available with this article online at http://www.immunity.com/cgi/content/full/22/3/285/DC1/.

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